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Measurements of Several Metallic Elements and Matrix Metalloproteinases (MMPs) in Saliva from Patients with Taste Disorder

Mamoru Watanabe[1], Masumi Asatsuma[1], Akihiro Ikui[2], Minoru Ikeda[2], Yoshiaki Yamada[1], Shuichi Nomura[3] and Atsuko Igarashi[1]

[1]Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences, Gakkocho Dori 2-5274, Niigata City, Niigata 951-8514, Japan, [2]Department of Otorhinolaryngology, Nihon University School of Medicine, Gakkocho Dori 2-5274, Niigata City, Niigata 951-8514, Japan and [3]Department of Oral Health Science, Niigata University Graduate School of Medical and Dental Sciences, Gakkocho Dori 2-5274, Niigata City, Niigata 951-8514, Japan

Correspondence to be sent to: Atsuko Igarashi, Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan. e-mail: atsuko@dent.niigata-u.ac.jp

Abstract

We have measured and compared several metallic elements and matrix metalloproteinases (MMPs) in saliva from patients with taste disorder and healthy subjects. Stimulated whole saliva was collected from 20 patients and 35 healthy subjects. Inductively coupled plasma mass spectrometry (ICP-MS) was used for the determination of metallic elements in saliva. Amounts of MMP-1, MMP-3, MMP-9 and IL-1α, IL-6 in saliva were measured using an enzyme-linked immunosorbent assay systems. Zinc in the serum was determined by flame atomic absorption spectrometry. Our results provide evidence that levels of zinc, manganese and the amount of MMP-3 in saliva are significantly decreased in the patients with taste disorder compared to the healthy subjects; Zn (p.p.b.): healthy subjects 79.8 ± 42.6, patients 47.22 ± 17.1, ($P < 0.001$), Mn (p.p.b.): healthy subjects 4.48 ± 2.46, patients 2.78 ± 1.23, ($P < 0.004$), MMP-3 (ng/ml), healthy subjects 0.820 ± 0.417, patients 0.594 ± 0.179 ($P < 0.01$). In contrast, copper is significantly increased in the patients; Cu (p.p.b.): healthy subjects 34.5 ± 13.5, patients 45.9 ± 20.8 ($P < 0.049$). These differences may be closely related with this disease. ICP-MS is an easy and accurate instrument for measurements of salivary metallic elements and may be useful in establishing a diagnosis of taste disorder.

Key words: copper, manganese, MMP-3, saliva, taste disorder, zinc

Introduction

It has been estimated that ~140 000 people per year in Japan develop taste disorder, which is said to be one of the symptoms of zinc deficiency (Tomita, 1998).

Saliva is necessary for taste, although its function in terms of taste has not yet been fully elucidated. Similarly, zinc plays an important role in taste perception (Henkin, 1975), and its deficiency is a common problem among the elderly. This deficiency is related to taste disorder, growth failure and impaired wound healing (Rundles, 1978). Some studies have already reported that carbonic anhydrase VI (a zinc metalloprotein) is decreased in patients with taste disorder (Henkin *et al.*, 1975; Thatcher *et al.*, 1998). In addition, zinc metal ions are necessary for the activity of many enzymes (Coleman, 1992).

Matrix metalloproteinases (MMPs) are related to the zinc hydrolysis components of the extracellular matrix (ECM). These proteinases play a central role in many biological processes, such as embryogenesis, normal tissue remodeling, repair and destruction. In our previous reports, we found that taste disorder was related to the decrease of tissue inhibitor of metalloproteinase (TIMP)-1 in saliva and that of serum zinc levels, and this was improved by zinc administration (Igarashi *et al.*, 2001; Monya *et al.*, 2003).

In this study, we measured and compared salivary metallic elements and MMPs for patients with taste disorder and healthy subjects.

Materials and methods

Subjects

The patient group consisted of 11 men and 9 women (mean age 64.8 ± 7.4 years) who were all diagnosed with taste

disorder by gustatory tests in the Outpatient Clinic of Taste Disorder, Nihon University Medical Hospital and Niigata University Dental Hospital. They were suspected of suffering from taste disorder due to zinc deficiency. Patients with psychogenic taste disorder, flavor disorder or systemic disease were excluded from this study. Healthy subjects were 35 volunteers (15 men, 20 women, mean age 67.2 ± 9.5 years) with normal gustatory acuity.

All the patients and healthy subjects had given their informed consent, and the Declaration of Helsinki (September 1989) was followed throughout the study.

Collection of saliva

Mixed saliva, which was secreted by the stimulation of chewing tasteless and odorless paraffin gum for 5 min, was collected (Table 1).

Immediately after the collection, the saliva sample was transferred to clean microtubes and then centrifuged at 12 000 r.p.m. for 5 min to remove the precipitate. The supernatant was frozen at −80°C until assay.

Gustatory test

All patients were diagnosed by the use of the whole mouth gustatory test described by Maes *et al.* (2002). Four test substances (sucrose, NaCl, HCl, urea) were used and three solutions of different concentrations were prepared for each test substance. No. 1 of each test solution contained plain water. The severity of hypogeusia was rated on a scale grade from 2 (normal) to 4 (severe) based on taste thresholds of four different tastes (Table 2).

Table 1 Age and salivary flow rates in the healthy subjects and patients with taste disorder

	Healthy subjects	Patients	*P*-value	Test
Age (years)	67.2 ± 9.5	64.8 ± 7.4	0.629	Mann–Whitney *U*-test
Salivary fluid (ml/5min)	6.0 ± 3.0	7.0 ± 4.5	0.325	Welch *t*-test

*P values < 0.05 were considered statistically significant.

Table 2 The concentrations (mM) of the solutions for the whole mouth gustatory test

Taste Substance	Grade				Taste type
	1	2	3	4	
Sucrose	0	60	100	200	sweet
NaCl	0	60	150	300	salt
HCl	0	15	30	60	sour
Urea	0	200	500	1000	bitter

The procedure of gustatory test was as follows: first, each 1 ml of these substances was given to the subjects in a random order. Then they were asked to judge the taste of each substance without swallowing. The subjects always rinsed their mouth after the degustation of each solution.

Subjects who had been rated grade 2 were assigned to the healthy subject group and those who had been rated grades 3–4 to the patient group.

Collection of serum and determination of serum zinc level

Sera were obtained from patients with taste disorder and from healthy subjects. They were always collected between 9.00 and 12.00 a.m. in the morning. After collection, the blood samples were transferred to zinc-free test tubes. The level of zinc in serum was measured by flame atomic absorption spectrometry.

Measurements of several metallic elements in saliva

An Agilent HP 4500 inductively coupled plasma mass spectrometry (ICP-MS) system (Yokogawa Analytical Systems Inc., Tokyo, Japan) was used for the measurements of the levels of metallic elements (Mg, Ca, Ga, Mn, Fe, Cu, Zn) in saliva. In this system, Ga was used as the internal standard element. The standard solutions were prepared from Kanto Chemicals, Tokyo, Japan (Mg, Ca, Ga: 1000 mg/l, Mn, Fe, Cu, Zn: 100 mg/l). Pure water (Milli-Q) and nitric acid (Kanto Chemicals) were needed and were prepared for this method. The internal standards were applied to each saliva sample and then the samples were diluted 1:15 (v/v) with 1 mM nitric acid. ICP-MS operating conditions are shown in Table 3.

Measurements for total amounts of MMP-1, MMP-3 and MMP-9 in saliva

The active forms of MMPs (MMP-1: 41 kDa, MMP-3: 45 kDa, MMP-9: 67 kDa) in saliva were present in too small an amount to be detected, so we needed to measure the total amounts (pro and active forms) of MMP-1, MMP-3 and MMP-9 using enzyme-linked immunosorbent assay (ELISA).

The total amounts of MMP-1 and MMP-9 were measured by using the Biotrak ELISA system (Amersham Biosciences, Bucks, UK). The detection limits were 6.25 and 4 ng/ml for MMP-1 and MMP-9 respectively. The total amount of MMP-3 was measured using the MMP-3 Biotrak Activity Assay System (Amersham Biosciences). The MMP-3 detection limit was 0.25 ng/ml.

Table 3 ICP-MS operating conditions

RF power	Sampling depth	Ar carrier gas	Ar auxiliary gas	Ar plasma gas	Sample uptake rate
1150 W	6.5 mm	1.2 l/min	1.0 l/min	15.5 l/min	0.5 ml/min

Measurements of IL-1α and IL-6 in saliva

The amounts of salivary IL-1α and IL-6 were measured by Cytokine ELISA kits (American Research Products and R&D Systems, USA). The detection limits for IL-1α and IL-6 were 8 and 0.156 pg/ml respectively.

Statistical analysis

Data were shown as mean ± SD and were statistically evaluated by the Mann–Whitney U-test, Student's *t*-test and Welch's test. $P < 0.05$ was regarded as statistically significant. The Mann–Whitney *U*-test was only applied for the comparison of ages in the two groups, because of the non-normal data distribution. On other data, when the equalities of the variances of two populations were confirmed, Student's *t*-test was used, and when they were not equal, Welch's test was employed.

Results

Zinc levels in serum and saliva

Serum zinc levels tended to decrease in the patients, although not significantly. However, the zinc level of saliva significantly decreased in the patients with taste disorder compared to the healthy subjects (Table 4).

The levels of other elements in saliva

The level of manganese in saliva was significantly lower in the patients compared to the healthy subjects. In contrast, copper in saliva was significantly higher in the patients compared to the healthy subjects. Other elements, such as Mg, Ca, Fe, showed no significant differences between the groups (Table 5).

Table 4 Serum and salivary zinc levels in the healthy subjects and patients with taste disorder

	Healthy subjects	Patients	*P*-value	Test
Serum Zn (p.p.b.)	830.9 ± 156.2	781.1 ± 91.1	0.157	Welch's *t*-test
Salivary Zn (p.p.b.)	79.8 ± 42.6	47.2 ± 17.1	0.001*	Welch's *t*-test

*P values < 0.05 were considered statistically significant.

Table 5 Metallic element levels in saliva in the healthy subjects and patients with taste disorder

	Healthy subjects	Patients	*P*-value	Test
Mn (p.p.b.)	4.48 ± 2.46	2.78 ± 1.23	0.004*	Welch's *t*-test
Cu (p.p.b.)	34.5 ± 13.5	45.9 ± 20.8	0.049*	Welch's *t*-test
Mg (p.p.m.)	1.86 ± 0.952	1.83 ± 0.782	0.93	Student's *t*-test
Ca (p.p.m.)	22.7 ± 5.54	24.9 ± 8.28	0.29	Student's *t*-test
Fe (p.p.b.)	122 ± 42.2	135 ± 17.4	0.16	Welch's *t*-test

*P values < 0.05 were considered statistically significant.

Amounts of MMPs in saliva

The total amount of MMP-1 was not significantly different between patients and healthy subjects. As for MMP-9, its total amount tended to increase in the patients although not significantly. On other hand, the total amount of MMP-3 was significantly decreased in the patients with taste disorder compared to the healthy subjects (Table 6).

Amounts of ILs in saliva

There was no significant difference for both the IL-1α and IL-6 of the patients and healthy subjects, although, IL-6 in particular showed a tendency to be decreased in the patients (Table 7).

Discussion

Zinc levels in serum and taste disorder

At present, the measurement of serum zinc is widely used as an indicator of taste disorder. Several reports have already suggested that a decrease in serum zinc is closely related to taste disorder (Henkin *et al.*, 1975, 1999). In addition, zinc administration improved taste disorder and recovered serum zinc levels (Sakai *et al.*, 2002; Sato and Mikami, 2002; Monya *et al.*, 2003). Moreover, in animals placed on a low-zinc diet, the taste bud cell group was injured and receptor cells disappeared or decreased in number, resulting in the development of taste disorder (Kobayashi and Tomita, 1986; Goto *et al.*, 2001). However, it has been suggested that zinc supplementation produces neither improvement of taste disorders nor increment of plasma zinc levels (Matson *et al.*, 2003).

Taste disorders might be rather correlated with the variation of zinc levels in saliva than in the serum (Henkin *et al.*, 1975, 1999). Serum zinc may be kept at a certain concentration at homeostasis. On the other hand, the zinc in saliva is considered to be greatly influenced by intake. Therefore, serum zinc measurements may prove to be of limited diagnostic value.

Salivary zinc and taste disorder

In this study, zinc levels in whole saliva significantly decreased in the patients. This data was determined by ICP-MS and this decrease may be evidence of taste disorder due to zinc deficiency. At present, as the salivary zinc level is very low, it is difficult to measure with flame aspiration atomic absorption spectrometry. Therefore, previous studies (Henkin *et al.*, 1975, 1999) have used flameless atomic absorption spectrometry to measure zinc levels in parotid saliva. Recently, ICP-MS has allowed the measurement of salivary zinc and other multiple elements more easily and accurately. In the future, this method may help to determine salivary zinc and other metallic elements on a common basis as a clinical evaluation for taste disorder.

Table 6 Salivary MMP-1, MMP-3 and MMP-9 amounts in the healthy subjects and patients with taste disorder

	Healthy subjects	Patients	*P*-value	Test
MMP-1 (ng/ml)	3.42 ± 1.12	2.87 ± 1.10	0.099	Student's *t*-test
MMP-3 (ng/ml)	0.820 ± 0.417	0.594 ± 0.179	0.01*	Welch's *t*-test
MMP-9 (ng/ml)	3.80 ± 2.44	5.13 ± 2.08	0.057	Student's *t*-test

*P values < 0.05 were considered statistically significant.

Table 7 Salivary IL-1α and IL-6 levels in the healthy subjects and patients

	Healthy subjects	Patients	*P* value	Test
IL-1α (pg/ml)	436.6 ± 417.1	470.8 ± 506.7	0.794	Student's *t*-test
IL-6 (pg/ml)	7.527 ± 10.14	4.054 ± 4.200	0.101	Welch's *t*-test

*P values < 0.05 were considered statistically significant.

Metallic elements in the saliva and serum

The new findings concerning manganese and copper levels in saliva suggest that the variation of these elements may be involved in taste disorder. Manganese and copper in saliva were significantly lower and higher, respectively, in patients with taste disorder than in healthy subjects.

Manganese participates in numerous metabolic responses and is a constituent of some enzymes such as manganese superoxide dismutase (MnSOD) and manganese catalase (Mn-CAT). Several diseases in humans have been linked to possible disturbances in manganese metabolism. However, research in this area is ongoing and inconclusive at this time (Finley and Davis, 1999).

The copper is a trace element and, like manganese, is important for the function of many cellular enzymes (Tapiero *et al.*, 2003). Traditionally, copper has been considered to antagonize zinc metabolism (Prasad *et al.*, 1978; Brewer *et al.*, 1989; Askari *et al.*, 2003), but a recent report has criticized this concept (Milne *et al.*, 2001). However, our clinical observations (data not shown), have found that the patients with low zinc level in serum corresponded usually to relatively high copper serum levels, and thus support the traditional concept.

Because the populations in this study are too small, a comparison between genders was not performed. Further studies on larger populations are needed to clarify the differences between males and females in the level of various metallic elements and MMPs in saliva.

Salivary MMPs

In this study, MMP-1 and MMP-9 were not significantly different between the patients and healthy subjects. In contrast, MMP-3 significantly decreased in the patients. For salivary MMPs, there are a few reports for periodontitis and Sjogren's syndrome (SS). The latter is known to relate to taste disorder. In our previous report, the MMP-9 level in saliva of primary SS patients was significantly higher than that in healthy subjects. Moreover, the ratio of MMP-9 to the tissue inhibitor of metalloproteinases-1 (TIMP-1) increased significantly in the primary SS patient group (Asatsuma *et al.*, 2004). MMPs are important components in many biological and pathological processes because of their ability to degrade ECM components. Moreover, MMPs are believed to play a key role in the tissue destruction accompanying inflammatory diseases such as periodontitis (Makela *et al.*, 1994).

In this study, MMP-3 was significantly decreased in patients ($P < 0.01$). MMP-3 (stromelysin-1) digests ECM components such as collagens III, IV, IX, X and fibronectin, and activates proMMPs (Visse and Nagase, 2003).

In addition, it has been previously reported that MMP-3 cleaves heparin binding epidermal growth factor (HB-EGF) at a specific site (Suzuki *et al.*, 1997) and Umeda *et al.* (2001) have suggested that HB-EGF is a crucial regulator of epithelial morphogenesis during organ development, highlighting the importance of its processing by metalloproteinases. Moreover, the development of the anterior taste bud requires epidermal growth factor receptor (EGFR) in mice (Sun and Oakley, 2002) and zinc-induced EGFR phosphorylation through the extracellular release of HB-FGF, which was mediated by MMP-3 (Wu *et al.*, 2004). Therefore, MMP-3 may serve in the continuous regeneration of taste buds.

Next, we tried to determine the active form of MMP-3 by Western blotting and casein-zymography. However, because of the small amount of MMP-3 in saliva, its active form was not detectable (data not shown). Further studies are necessary to establish a relationship between MMPs, TIMPs and zinc in saliva from patients with taste disorder.

In addition, to examine the inflammatory condition, we measured salivary IL-1α and IL-6 and found no differences between patients and healthy subjects.

The present results may provide new knowledge about the taste disorder and can serve as a basis for a biochemical approach to the study of the etiology and treatment of this disease.

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